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Biochemical and Biophysical Research Communications 334 (2005) 764–768
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The transcription factor Stat3 is dispensable for pancreatic β-cell development and function ☆

Ji-Yeon Lee, Lothar Hennighausen *

Laboratory of Genetics and Physiology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA

Received 14 June 2005
Available online 11 July 2005

Abstract

The transcription factor Stat3 is activated by multiple cytokines, including leptin and those signaling through the gp130 receptor. In two independent studies, mice in which the Stat3 gene was inactivated using a RIP-Cre transgene led to glucose intolerance, defects in early-phase insulin secretion, and mild obesity [S. Gorogawa, Y. Fujitani, H. Kaneto, Y. Hazama, H. Watada, Y. Miyamoto, K. Takeda, S. Akira, M. Magnuson, Y. Yamasaki, Y. Kajimoto, M. Hori, Insulin secretory defects and impaired islet architecture in pancreatic beta-cell-specific STAT3 knockout mice, Biochem. Biophys. Res. Commun. 319 (2004) 1159; Y. Cui, L. Huang, F. Elefteriou, G. Yang, J. Shelton, J. Giles, O. Oz, T. Pourbahrami, C. Lu, J. Richardson, G. Karsenty, C. Li, Essential role of STAT3 in body weight and glucose homeostasis, Mol. Cell. Biol. 24 (2004) 258]. However, since the RIP-Cre transgene is also expressed in the hypothalamus, and thereby Stat3 was deleted from neurons expressing the leptin receptor, it was not clear as to which of the metabolic defects were due to the loss of Stat3 from β-cells or the hypothalamus. We have addressed this issue through the inactivation of Stat3 from pancreatic β-cells using a Pdx1-Cre transgene. Complete loss of Stat3 was observed in islets from mice, which carry two floxed Stat3 alleles and the Pdx1-Cre transgene. However, these mice did not develop glucose intolerance or obesity over a period of 6 months, demonstrating that Stat3 is dispensable for the generation and physiology of β-cells. Similarly, mice that express only the Pdx1-Cre transgene display a normal physiology. In contrast, mice that expressed only the RIP-Cre transgene developed glucose intolerance as early as 6 weeks of age. The finding that RIP-Cre transgenic mice in a C57B/6 dominated background develop glucose intolerance is important as this line has been used in several studies.
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Keywords: Stat3; Pdx1-Cre; RIP-Cre; Islets of Langerhans; β-cells; Glucose homeostasis

Signal transducers and activators of transcription (STATs) are widely expressed transcription factors that are activated by cytokines and subsequently initiate a plethora of biological responses. One member of this family, the ubiquitously expressed Stat3, is activated by a variety of cytokines, including EGF, IL-6, OSM, LIF, leptin, prolactin, and by non-receptor tyrosine kinases. Some of these cytokines have been shown to contribute to the proliferation, survival, and function of pancreatic β-cells in in vitro and gain-of-function experiments [3–6]. Since loss of Stat3 does not yield viable mice [7], Cre-loxP-based approaches were used by two research groups to explore the function of Stat3 in pancreatic β-cells [1,2]. These investigators generated mice that carried two floxed Stat3 alleles [7] and a Cre transgene [8,9] under control of the rat insulin promoter (RIP-Cre). Both groups observed that these mice displayed glucose intolerance, a deficiency in early-phase

☆ *Abbreviations:* Stat, signal transducer and activator of transcription; JAK, Janus kinase; EGF, epidermal growth factor; IL-6, interleukin-6; OSM, oncostatin M; LIF, leukemia inhibitory factor; HGF, hepatocyte growth factor; HB-EGF, heparin-binding EGF-like growth factor.

* Corresponding author. Fax: +1 301 480 7312.
E-mail address: hennighausen@nih.gov (L. Hennighausen).

0006-291X/$ - see front matter. Published by Elsevier Inc.
doi:10.1016/j.bbrc.2005.06.162

insulin secretion, and also developed a mild obesity. Moreover, islet morphology was altered with α- and β-cells being interspersed. The RIP-Cre transgene used in these studies was also expressed in the hypothalamus [9] and Stat3 deletion occurred in leptin receptor-positive neurons, suggesting, and as pointed out by these investigators [1,2], that some of the defects observed in these mice were non-autonomous of the pancreatic β-cells. In one study, this was supported by transplant experiments in which wild-type islets failed to reverse the obesity and glucose intolerance in these mice [2]. However, the transplantation was performed in 12-week-old mice, a time when obesity and glucose tolerance had been fully established. It was therefore not clear to what extent Stat3 in the hypothalamus or the pancreatic β-cells contributed to the defects observed in these mice.

In order to definitively address the question of to what extent Stat3 in β-cells is required for their function and glucose homeostasis, we now inactivated the Stat3 gene [10] in β-cells using the Pdx1-Cre transgene [11]. This transgene is expressed in precursor cells that give rise to the α- and β-cell lineages and deletion of floxed genes is obtained in β-cells [11]. In contrast to the RIP-Cre mice used in other studies, Cre expression in these mice has not been observed in the hypothalamus. Moreover, we also investigated whether the expression of Cre itself can alter the physiology of β-cells and the metabolism of mice. For this purpose, mice that expressed either the RIP-Cre or Pdx1-Cre transgene were compared.

Materials and methods

Animals and genotyping. The Stat3$^{fl/fl}$ mice used in this study carry a Stat3 gene in which the exons 16–21 are bracketed by loxP sites [10]. Loss of these exons, which includes the SH2 domain, results in the complete absence of Stat3 [10,12]. Stat3$^{fl/fl}$ mice were bred with a mouse containing the Pdx1-Cre transgene [11] (called PC throughout the manuscript). Stat3$^{fl/+}$; PC mice, which had been generated by crossing Pdx1-Cre mice with Stat3$^{fl/fl}$ mice, were mated with Stat3$^{fl/fl}$ to generate Stat3$^{fl/fl}$; PC mice. All experiments were performed by using progenies of female Stat3$^{fl/fl}$ mice crossed with male Stat3$^{fl/fl}$; PC mice. Mice were maintained on a 12-h light, 12-h dark cycle and fed water and standard diets. NIH-07 Rodent chow was obtained from Zeigler. It contains at least 23.5% protein and at least 4.5% fat. The NIDDK Animal Care and Use Committee approved all procedures and studies were conducted in accordance with National Institutes of Health guidelines. Genotyping was performed by PCR amplification of tail DNA from each mouse at 3 weeks of age. The following primers were used for genotyping of the *Stat3* floxed allele: primer 1 (5′-GAA GGC AGG TCT CTC TGG TG-3′) and primer 2 (5′-AGG CTG CCA ACA GCC ACT GCC-3′). This primer pair amplifies a fragment of 140 bp from the wild-type Stat3 allele and a 260 bp fragment from floxed Stat3 allele. Primers for the Cre transgene were 5′-TTG AAA CAA GTG CAG GTG TTC G-3′, which binds in the Pdx1 gene promoter, and 5′-CCA TGA GTG AAC GAA CCT GGT CG-3′, which binds in the Cre sequence. This pair amplifies a 730 bp Pdx1-Cre-specific fragment.

To detect the recombined Stat3 allele at the level of genomic DNA, pancreatic and hypothalamic tissues were isolated from Stat3$^{fl/f}$, Stat3$^{fl/+}$; PC and Stat3$^{fl/fl}$; PC mice. By PCR, a 370 bp fragment representing the recombined allele was detected with primers 2 and 3 (5′-CAG AAC CAG GCG GCT CGT GTG-3′). All PCRs were performed at 94 °C for 2 min, followed by 30–35 cycles of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s.

Immunohistochemical analyses. Pancreatic specimens were dissected from fed mice and fixed in 4% paraformaldehyde/PBS solution overnight at 4 °C. Five-micrometer longitudinal sections of paraffin-embedded tissue were rehydrated with xylene followed by decreasing concentrations of ethanol. Antigen retrieval was performed by heat treatment with an antigen unmasking solution (Vector Laboratories), and tissue sections were blocked for 30 min in PBS-Tween (PBST) containing 3% goat serum. Sections were incubated with anti-Glucagon (Sigma Cat. No. G2654) and anti-insulin (Dako Cat. No. A0564, Mississauga, Canada) antibodies. The primary antibodies were allowed to bind overnight at 37 °C. Secondary antibodies were from Molecular Probes (Eugene, OR) and included goat anti-mouse immunoglobulin G (IgG) conjugated to Alexa Fluor 594 and goat anti-guinea pig IgG conjugated to Alexa Fluor 488. Sections were incubated in the dark for 30 min, washed twice in PBST, and mounted with Vectashield (Vector Laboratories). Immunofluorescence was viewed under an Olympus BX51 microscope (Olympus America, Melville, NY) and images were captured with a Nikon DXM1200 digital camera (Nikon, Melville, NY).

Islet isolation and Western blotting. To determine the amount of Stat3 protein in islets, islets were isolated from Stat3$^{fl/f}$ and Stat3$^{fl/fl}$; PC mice by collagenase digestion and differential centrifugation through Ficoll gradients. Whole islets from three animals were lysed in 100 μl of 2× lysis buffer (40 mM Tris–Cl, pH 8.0, 0.28 M NaCl, 20% glycerol, 2% NP-40, 4 mM EDTA, pH 8.0, and 20 mM NaF) by grinding using pellet pestle (Kontes) and obtained supernatant by centrifugation. After quantification, 30 μg of protein per well was loaded on 8% protein gel. Primary antibodies used were rabbit anti-Stat3 (Cell Signaling, Cat. No. 9132) and mouse anti-actin (Chemicon Cat. No. MAB1501).

Glucose tolerance test. Glucose tolerance tests were performed in fasted animals after i.p. injections of glucose (2 g/kg body weight). Blood glucose values were measured immediately before and 15, 30, 60, and 120 min after glucose injection. All results are reported as means ± SEM for equivalent groups. Results were compared with independent *t* tests (unpaired and two-tailed) reported as *P* values.

Results and discussion

Inactivation of the mouse Stat3 gene in β-cells

To study the role of Stat3 in pancreatic β-cells, mice were generated that carried two Stat3 floxed alleles [10] and a Pdx1-Cre transgene [11] (Stat3$^{fl/fl}$; PC mice) (Fig. 1A). The presence of Stat3 was analyzed in isolated islets using Western blots (Fig. 1B). While Stat3 was abundant in isolated islets from control mice, it was completely absent in islets from Stat3$^{fl/fl}$; PC mice at 4 months of age (Fig. 1B). Immunohistochemical analyses of pancreatic sections from control and Stat3$^{fl/fl}$; PC mice (Figs. 1C and D) showed that overall distribution of islet and their architecture was normal.

Loss of Stat3 from β-cells does not result in glucose intolerance or obesity

To examine the impact of the loss of Stat3 on the response to an acute glucose challenge, glucose disposal

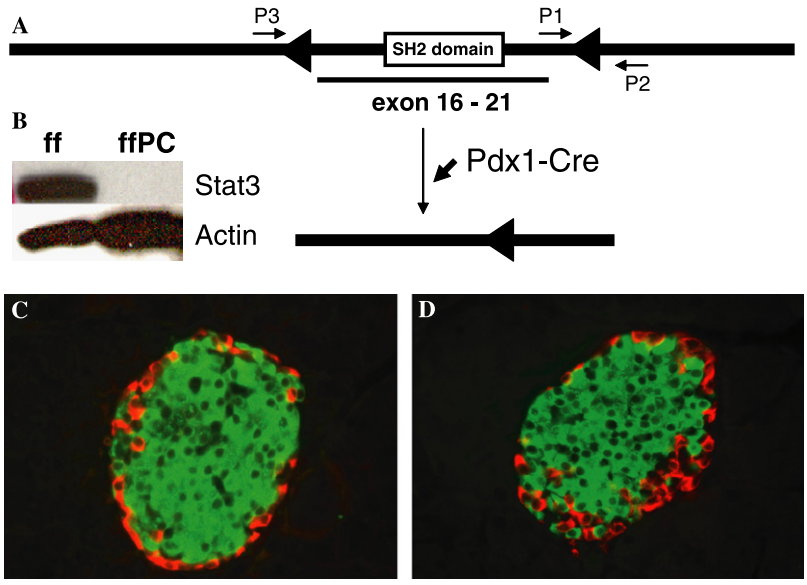

Fig. 1. Targeted disruption of the Stat3 gene and assessment of Stat3 deletion in β-cells of Stat3$^{fl/fl}$; PC mice. (A) Diagram of the Stat3 allele targeted with loxP sites and upon Cre-mediated recombination in Stat3$^{fl/fl}$; PC mice. Exons 16–21 of Stat3 were flanked by two loxP sites. P1, P2, and P3 represent primers for detection of floxed or recombined Stat3 alleles. (B) Western blot demonstrating the absence of Stat3 in the pancreatic islets. Thirty micrograms of islet protein was loaded in each well. Actin staining was performed on the same membrane to control for loading. (C,D) Fluorescence immunohistochemical analysis of insulin (green) and glucagon (red) in Stat3$^{fl/fl}$ (C) and Stat3$^{fl/fl}$; PC (D) mice. Pancreatic sections were obtained from 6-month-old mice.

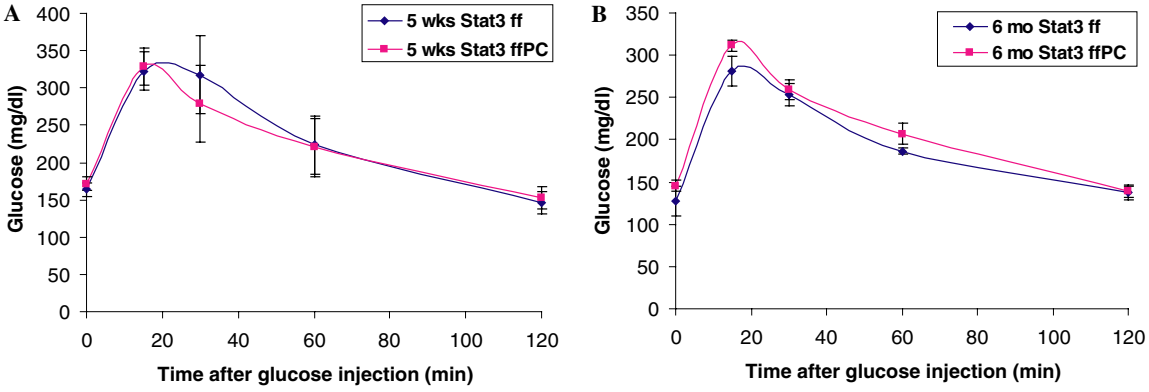

Fig. 2. Glucose tolerance in Stat3$^{fl/fl}$; PC mice. Glucose tolerance tests were performed on fasted mice after an i.p. injection of 2 g/kg at either 5 weeks (A) or 6 months (B) of age. Blood glucose concentrations were determined on blood samples obtained by tail biopsy at the indicated time points. Results are expressed as average blood glucose levels ± SEM of 6–8 males of each group. There were no significant differences between Stat3$^{fl/fl}$; PC and control mice at any given time point.

was assessed in fasted mice by glucose tolerance tests. Glucose levels in fasting Stat3$^{fl/fl}$; PC males prior to the administration (ip) of glucose were normal at the ages of 5 weeks and 6 months (Figs. 2A and B). Glucose levels from wild-type and Stat3$^{fl/fl}$; PC mice were indistinguishable at all time points after glucose injection. Moreover, the weight of control and mutant mice was comparable (Fig. 3).

In many settings cytokine-induced Stat3 activation occurs through the gp130 receptor, which is the shared subunit of different cytokine receptors. Notably, IL-6 signals through gp130 and controls insulin secretion in β-cells in vitro [13]. Inactivation of the gp130 gene generally leads to a loss of Stat3 signaling [14,15]. In order to inactivate the gp130 gene in β-cells, mice were generated that carried two floxed gp130 allele [14] and the Pdx1-Cre transgene (gp130$^{fl/fl}$; PC mice). These mice did not develop glucose intolerance and no defective islets were observed (data not shown).

The experiment presented excludes roles for the transcription factor Stat3 and cytokines relying on the gp130 chain in the development and function of mouse pancreatic β-cells. Our results further support that previously recognized defects in β-cells from mice, in which the Stat3 gene had been deleted using the RIP-Cre transgene, are likely to be of indirect nature. Most notably,

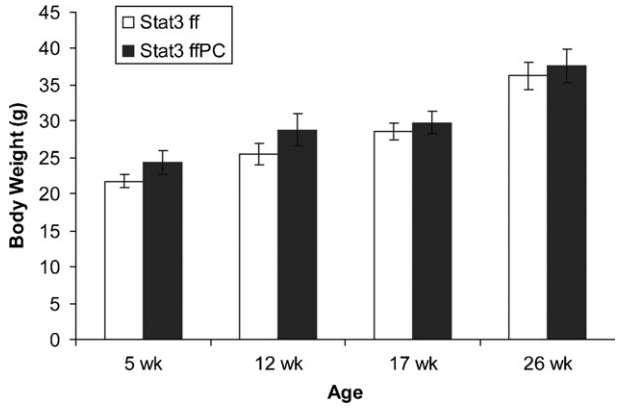

Fig. 3. Body weight of Stat3$^{fl/fl}$; PC and control (Stat3$^{fl/fl}$) mice at 5 weeks, 12 weeks, 17 weeks, and 26 weeks of age. Each data point is presented by mean ± SEM of 6–8 females of each group. Body weights of the control groups were comparable through whole life. Similar results were observed in male mice.

a key difference between the Pdx1-Cre and the RIP-Cre transgenes lies in the fact that the latter one is expressed in the hypothalamus [9] including neurons expressing the leptin receptor. However, it is also possible that the RIP-Cre transgene in certain mouse strain backgrounds might exert some adverse effects on the metabolism of pancreatic β-cells.

RIP-Cre but not Pdx1-Cre mice develop glucose intolerance

Mice that carry two floxed Stat3 alleles and a RIP-Cre transgene developed glucose intolerance and obesity [1,2]. These studies used floxed Stat3 mice as controls, but not RIP-Cre mice by themselves. To establish whether the RIP-Cre gene could contribute to the altered physiology of mice, glucose tolerance tests were performed on RIP-Cre mice distributed by the Jackson Laboratory, Pdx1-Cre and wild-type mice at 6 weeks and 4 months of age (Fig. 4). While glucose levels from wild-type and Pdx1-Cre mice were indistinguishable at all time points after glucose injection, RIP-Cre mice displayed glucose intolerance already at 6 weeks of age. The glucose intolerance was exacerbated at 4 months of age, when glucose levels reached 450 mg/dl 15 min after injection, while those seen in age-matched Pdx1-Cre mice were approximately 300 mg/dl (Fig. 4). RIP-Cre transgenic mice did not develop obesity as their body weight was comparable to that of wild-type mice up to 6 months of age (data not shown). These experiments support the notion that the glucose intolerance observed in RIP-Cre mice alone could contribute to the phenotype published for Stat3/RIP-Cre mice [1,2].

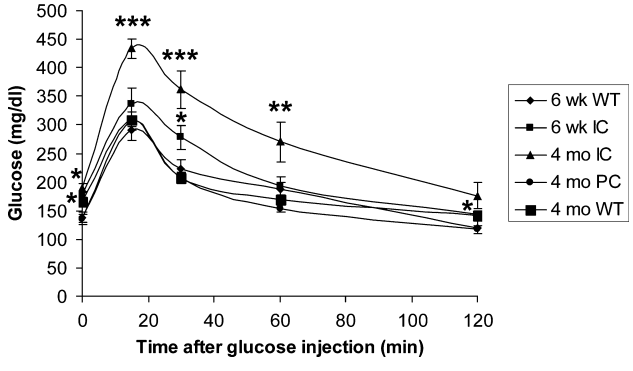

Fig. 4. Glucose tolerance in wild-type (WT), RIP-Cre (IC), and Pdx1-Cre (PC) mice. Six-week-old WT and IC mice, 4-month-old WT, IC, and PC mice were tested. Each group contained 4–8 female mice. Glucose levels of IC and PC mice were compared with levels of same-aged WT mice for statistical t test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

These RIP-Cre mice were generated in 1999 [8] and have been used by at least 17 investigators to inactivate at least 14 different genes. Studies with these mice have yielded profound insight into genetic pathways controlling β-cell development and function as well as metabolism controlled by the hypothalamus. Glucose homeostasis in RIP-Cre mice appeared to be unaffected in the original mixed strain background. However, the RIP-Cre mice were subsequently bred into a pure C57B/6 background by the Jackson Laboratory, which also distributed the mice. While some investigators used RIP-Cre mice as controls in their studies, others did not. A detailed evaluation of these RIP-Cre mice and the studies they have been used in is forthcoming (Hennighausen, Lee, Ristow, White, and Magnuson, in preparation for JCI).

Acknowledgments

The authors thank David Levy for generously providing the floxed Stat3 mice and Doug Melton for the Pdx1-Cre mice. The authors also acknowledge Oksana Gavrilova (Mouse Metabolic Core facility, National Institute of Diabetes and Digestive and Kidney Diseases, National Institute of Health) for help in isolating islets; Dinesh Gautam for help in isolating hypothalamus (Molecular Signaling Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health) and Lloyd Webster for technical assistance.

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